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Introduction

Small, non-peptidal molecules which mimics the 3D structure of heregulin (HRG) binding domain may specifically block receptor-binding and inhibit the biological activities of the HRG and HRG receptor(s). We have used a structure-based strategy towards the discovery of small molecules as potential specific HRG antagonists. In this approach, pharmacophore (spatial arrangement of functional groups) models were constructed based upon the crucial residues in HRG that may be responsible for binding to its receptors, as well as the chemical nature and the three dimensional (3D) geometry of these residues in the 3D structure of HRG. HRG constitutes the HRG subfamily of EGF-related peptides that were originally isolated from human breast cancer cell and ras-transformed fibroblasts. HRG can stimulate proliferation and may function as an autocrine growth factor in transformed cells. Stable expression of HRG via transfection leads tumor formation in nude mice and might perform a role in progression to estrogen-independent tumor growth.

Our previous focus has been to design and develop a functional assay system to determine the mechanisms of action of the small molecules, and to discover more novel compounds with improved potency. We have achieved this goal through utilization of 32D model system in which the 32D cells have been transfected with individual or in combination of *erbB* receptors, and molecular modeling-assisted, rational design of new models based upon protein structure and these lead compounds, followed by biochemical and biological evaluations of these new candidates. Compounds that were able to block the function of HRG in binding and HRG-induced phosphorylation assays and HRG-dependent growth were considered as the **lead compounds**. Three classes of lead compounds were discovered and we have demonstrated proof of the principle that small molecules discovered through structure and computer pharmacophore search can block HRG binding and inhibit the biological activity of HRG receptors.

We have demonstrated proof of the principle that small molecules discovered through structure and computer pharmacophore model search can block HRG binding and inhibit the biological activity of HRG receptors. This type of small molecule HRG antagonists will undoubtedly have a great therapeutic potential to be used either by themselves or in combination with other conventional chemotherapeutics in the treatment of breast cancer. We believe that the combined efforts of this multi-disciplinary team will bring significantly improved small, non-peptidal molecule HRG antagonists into a phase I clinical trial in the near future.

Specific Introduction for This Report.

Targeted disruption of a clinically relevant, oncogenic protein with small molecules is a new and attractive strategy. Our 3D-database pharmacophore search technique, and functional assay of the lead compounds is an unique and effective approach. Several small molecule compounds that inhibits the PDGF or VEGF receptor kinase activity has entered phase I or phase II clinical trials for the treatment of solid tumors. Our program has unique advantages. First, several promising lead compounds have already been discovered. Second, these lead compounds are small, non-peptidal, drug-like molecules. In fact, they are all natural products. Third, we have assembled a team with extensive experience in molecular modeling, drug design, cell and molecular biology, and pre-clinical studies in breast cancer research. This provides us

unparalleled strength for discovery of lead compounds using our effective approaches and advancement of lead compounds into pre-clinical and phase I clinical studies.

This team has extensive experience in molecular modeling, 3D-database, drug design, molecular biology and breast cancer research. We have demonstrated proof of the principle that small molecules discovered through structure and computer pharmacophore model search can block HRG binding and inhibit the biological activity of HRG receptors. It is expected that our program will generate specific, potent HRG antagonists as pre-clinical or clinical candidates in the near future. This type of small molecule HRG antagonists will undoubtedly have a great therapeutic potential to be used either by themselves or in combination with other conventional chemotherapeutics in the treatment of breast cancer. We believe that the combined efforts of this multi-disciplinary team will bring significantly improved small, non-peptidal molecule HRG antagonists into a phase I clinical trial in the near future.

If this approach is successful, it is expected that our program will generate specific, potent HRG antagonists as clinical candidates in the near future. This type of small molecule HRG antagonists will undoubtedly have a great therapeutic potential to be used either by themselves or in combination with other conventional chemotherapeutics in the treatment of breast cancer.

This project also received support in part by the Lombardi Cancer Center, and the Developmental Project of SPOR program for Breast Cancer from the Lombardi Cancer Center for the past two years.

The lead compound Rifamycin has received a patent report from US Patent and Trademark Office (PCT/US97/21474).

Body of Report

There were three technical objectives in the original proposal. The technical objective 1 was to investigate mechanisms of action of small molecule drugs we have identified previously. We propose to utilize the 32D functional assay system to investigate: 1) whether these small molecule lead compounds specifically blocks the HRG binding to *erbB-4* homodimer and HRG-mediated biological effects via the *erbB-4* receptor; 2) whether these compounds act differently in blocking the binding of HRG to heterodimer of *erbB* receptors such as *erbB-2/3*, *erbB-1/3*, *erbB-1/4* and *erbB-2/4*; and 3) whether these compounds might interfere with other EGF-like proteins binding or signaling pathways, such as EGF, epiregulin, HRG (or NRG-3.

The technical objective 2 was to discover additional novel lead compounds with better potency. We propose to discover additional novel lead compounds with better potency through: 1) searching the analogs of the three lead compounds; 2) designing additional pharmacophore models based on the HRG \exists EGF domain binding components and two-binding sites model of HRG; 3) pharmacophore searching of NCI database and ACD database. The advantage of having molecules that mimic two distinct binding sites is that these molecules may achieve synergistic

effect. Furthermore, we may test additive and/or synergistic effect of compounds that mimic two bindings by combinational treatment or chemically linking two molecules.

The technical objective 3 was to investigate the biological activity, specificity and therapeutic efficacy of the lead compounds. We propose to investigate lead compounds that were tested for both activity and specificity in 32D function system for their biological activities in human breast cancer cell lines. We will then test the 2-3 most promising compounds on human breast cancer xenograft on nude mice and transgenic models for therapeutic efficacy. This objective has not been accomplished as the co-PI Dr Shaomeng Wang moved to University of Michigan, Department of Internal Medicine last September. My lab is also in the middle of moving to the same department by February 2002. We plan to complete the remaining objectives and submit the final report in a year.

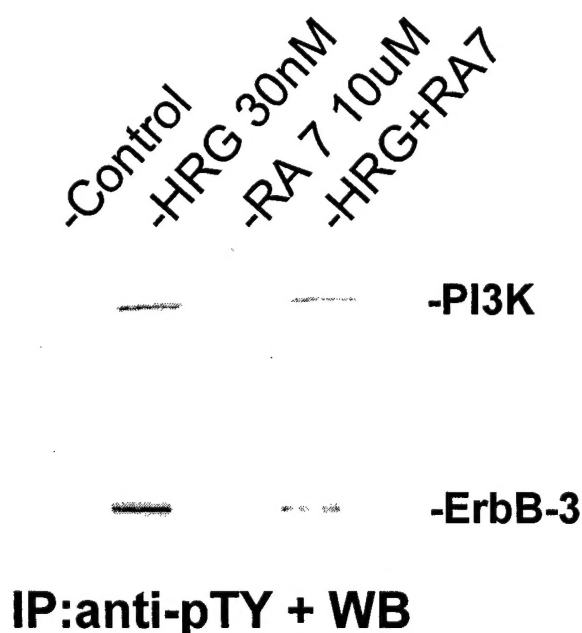
A search of the 3D-database of 206,000 compounds yielded 850 compounds, which were found to satisfy the pharmacophore query. The hydrophobicity of these 850 compounds was then examined and about 250 compounds were found to contain some hydrophobic moiety(ies) that could somewhat mimic those three crucial hydrophobic residues of HRG. One hundred-five compounds were tested and three classes compounds were identified as lead compounds in the functional assays.

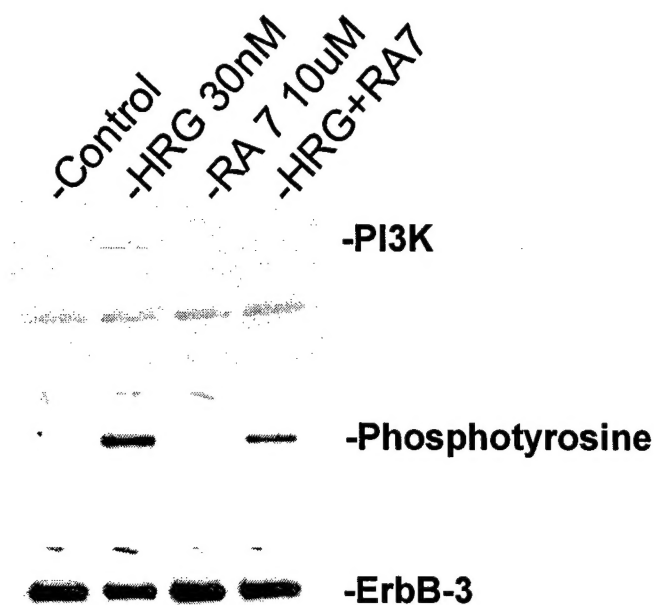
Most human cancer cells express more than one *erbB* receptors and ligands. Because different composition, expression and heterodimerization of various receptors might alter the binding affinity and/or internalization, it is difficult to define the exact correlation of the receptor expression and the antagonist activity of the small molecules. To overcome this difficulty, one could utilize a model cell system in which the same background cells are transfected with a particular growth factor and/or receptor as well as control genes. 32D cells from non-tumorigenic, murine hematopoietic cell line, are devoid of receptors for many growth factors (e.g., EGF, PDGF, *erbB*-2/3/4, KGF, IL-2, CSF-1, Met, Kit, etc.) and are strictly dependent on IL-3 for survival and proliferation. This IL-3 dependence, however, can be bypassed by the stimulation of signal transduction pathways initiated by the expression of specific growth factor receptors and the addition of the appropriate ligand to the culture medium. The IL-3 requirement could also be abrogated by oncogene-induced transformation such as *abl*, *src*. Compared with other commonly used mouse fibroblasts such as 3T3 cell line, there are two main advantages of the 32D model cell system. First, the 32D cells are devoid of many receptors, therefore, provide almost zero background of receptor autophosphorylation or cross-talks between receptors. Second, when 32D cells are transfected with a particular growth factor receptor, dual mitogenic and signal transduction pathways are created for the same transfectants expressing that receptor. For instance, 32D cells transfected with HRG receptor *erbB*-4 will proliferate in the presence of either HRG or IL-3. With this 32D cell model system, candidate compounds can be tested for their antagonist activity and specificity in both HRG-dependent and HRG-independent growth of the same cells. Using a model cell system which does not express *erbB* receptors, we have demonstrated for the first time that cells that express either *erbB*-4 or a combination of *erbB*-3 with *erbB*-2 exhibit enhanced response to heregulin chimerical toxin mediated cell killing. The heregulin-PE toxins have no activity on cells that express *erbB*-1/EGFR or *erbB*-2 receptor. When *erbB*-3 is expressed alone, heregulin-PE toxins have little or no activity. Thus, both the *erbB*-4 homodimer or the *erbB*-2 and *erbB*-3 heterodimers are the functional receptors for ligand

heregulin. We will utilize this model system to screen small molecule antagonists that act only on the heregulin. Compounds that act as like an agonist may also be identified if cultured in the absence of HRG. Furthermore, this system will allow us to determine if some of the compounds may preferentially block the heterodimer such as *erbB*-2/3, or *erbB*-1/3, and whether some of compound may have activity against the new HRG-like ligand such as HRG (.

Single or double transfection of 32D cells by the *erbB* receptors have been established through a collaboration with Dr. Y. Yarden, which allow testing of the inhibitory potency of candidate compounds on the specific expression of *erbB* receptors. 32D cells transfected with *erbB*-4 or *erbB*-2/3 will proliferate in the presence of either HRG or IL-3. With this 32D cell model system, candidate compounds can be tested for their activity and specificity in various *erbB* homo or heterodimer cells. More importantly, effect of a true small molecule antagonists can be reversed by addition of excess amount of HRG.

From current studies, we found that lead compound A1 inhibited only 32D/*erbB*-4 cells grow in the presence of HRG, but not in the presence of IL-3. SM A1 also does not inhibit EGF stimulated growth in 32D-EGFR cells. We believe that through this assay we will be able to identify compounds that interfere only with HRG signaling pathway.





IP:anti-erbB-3 + WB

In the biochemical studies, we have used the cells that express little phosphorylated receptors and stimulated phosphorylation of receptor by addition of HRG into the culture medium. However, at the presence of the HRG inhibitor RA7, the activation of the receptor is totally blocked. We further demonstrated such inhibition is an reversible event since the addition of extra amount of HRG could partially reverse the small molecule's effect. The total protein expression of the receptor was not affected by HRG or small molecule inhibitors.

More than 10-fold improvement on inhibition of receptor binding.

Screening of 30 more rifamycin analogs in dose-dependent receptor-binding assays were performed and four analogs were found to have IC_{50} about 1-10 μ M. This represent more than 10 fold improvement for initial rifamycin compounds in the receptor binding assay, which was between 100-200 μ M.

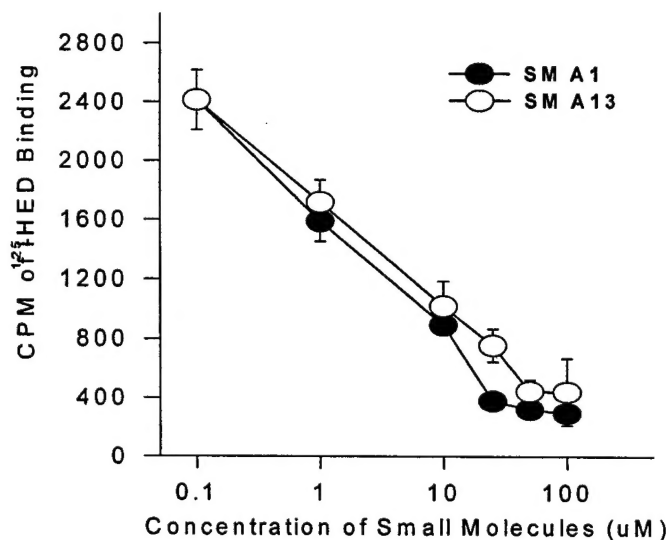
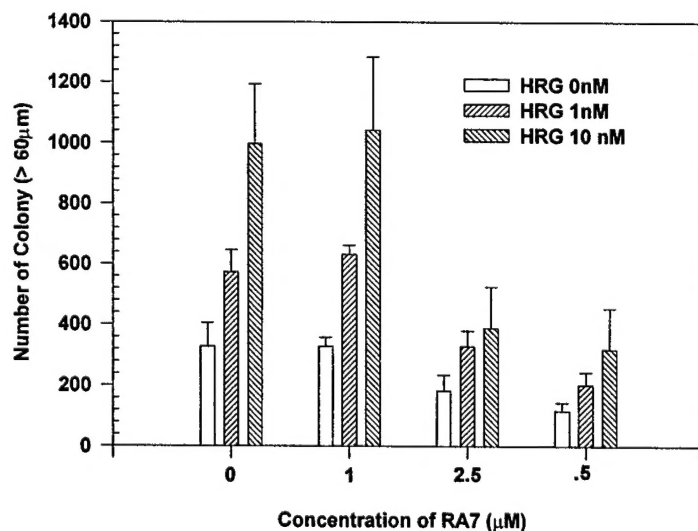


Fig. 2 Effect of Small Molecule on Heregulin Binding

Inhibition of HRG-induced soft-agar colony formation and reverse with addition of excessive amount of HRG.

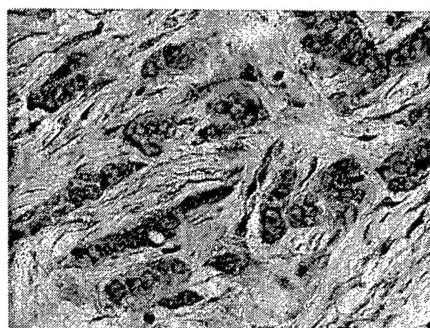
We have made recombinant HRG- Δ 1 which induce phosphorylation in a dose-dependent manner over range of 0.01 nM to 1 uM in MCF-7 or T47D cells. The recombinant HRG- Δ 1 can induce soft agar colony formation of MCF-7 in a dose-dependent manner. The ability of the small molecules to block HRG-induced soft-agar colony formation was tested by co-incubation



with 1nM, 10nM HRG and 1uM, 2.5uM and 5uM of lead compound SM-A1(RA7). An example of dose dependent inhibition of HRG-induced colony formation is shown. The specificity of HRG antagonist activity was evidenced by reverse of inhibition with excessive amount of HRG.

Elevated Expression of HRG in Human Breast Cancer Tissues.

A specific mAb that recognize HRG β in the paraffin tumor samples was found and used to carry out immunohistology staining of total 64 human breast cancer samples. Thirty one tumor samples (49%) were found to stain positively for HRG β in tumor cells, including the DCIS and invasive ductal carcinomas. Two examples of such staining of HRG β in human breast cancer cells were shown below.



Significance.

HRG constitutes the HRG subfamily of EGF-related peptides that were originally isolated from human breast cancer cell line MDA-MB-231, and ras-transformed Rat-1 fibroblasts. HRG can stimulate proliferation and may function as an autocrine growth factor in transformed mammary epithelial cells. Stable expression of ligand HRG via transfection leads tumor formation in nude mice and might perform a role in progression to estrogen-independent tumor growth. Furthermore, HRG induces *in vivo* lobuloalveolar development of mammary gland, and in MMTV-HRG transgenic mice, HRG induces persistence of terminal end buds and mammary adenocarcinoma, and 50% developed Harderian hyperplasia (a benign tumor). Clinically, elevated expression of HRG play a role in breast cancer growth and progression via autocrine loop and is associated with less favorable disease outcome. Thus, the discovery of these lead compounds represents an exciting and important step in the development of the small molecule, specific HRG antagonists as potential clinical candidates in the prevention and treatment of breast cancer.

Immunostaining studies show that *erbB-3* is overexpressed in breast cancer, and others. The expression of *erbB-4* is elevated in breast cancer cell lines, and we have found its overexpression in invasive ductal carcinoma and DCIS of breast but not in the nearby normal breast cells. More recently, three more heregulin-like genes, NRG2, NRG 3 and HRG (, interact with *erbB-3/4* or *erbB-4* only, have been reported. Our laboratory have been engaged in projects aimed at development of *erbB* receptor-mediated therapy in breast cancer. Expression of the EGFR, *erbB-2*, *erbB-3*, *erbB-4*, HRG and *in vivo* tumorigenicity of breast cancer cells and their response to the heregulin-PE toxins mediated killing have been well characterized.

Recently, it has been shown that it is indeed possible to discover and identify small molecules that can disrupt the protein-protein interactions. One example of such is published

recently by Li *et al.* in the discovery of the small molecule that effectively block the stable association between CD4 and MHC II protein. These small molecules appears to specifically target the CD4/MHC II complex and showed inhibition of immune responses in animal models and allograft transplant rejection. Therefore, these CD4 inhibitors may be developed as novel immunotherapeutics. We believe that the design and discovery of growth factor receptor antagonists will become a very active and fruitful research area in the near future.

Future Plan.

It is anticipated that we might discover and furthermore, synthesize lead compounds with potency and toxicity meet criteria for clinical trial studies in near future. We would then need to have the scale-up production with GMP standard to proceed with IND filing. Lombardi Cancer Center Developmental Therapeutic Program has run many clinical trials for novel anti-cancer drugs and will assist us in the design and execution of clinical trial study.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- ◆ Characterized the biological activity and specificity of small molecule antagonist of growth factor Heregulin in 32D model system;
- ◆ Biochemical and biological studies of the small molecule antagonist of HRG;
- ◆ Provided the proof-of-concept for the small molecule inhibitor studies of HRG interruption and also the success of funding from the Developmental Project of the SPORE Program from the Lombardi Cancer Center.
- ◆ Discovered more potent small molecule antagonist for HRG with further cell based inhibition activities.
- ◆ Report of one allowed patent application of small molecule antagonist of HRG

REPORTABLE OUTCOMES: Provide a list of reportable outcomes to include:

- manuscripts, abstracts, presentations;

Gao Y, Voigt J, Wu JX, Yang D*, Burke TR Jr. Macrocyclization in the design of a conformationally constrained Grb2 SH2 domain inhibitor. *Bioorg Med Chem Lett* 2001 Jul 23;11(14):1889-92

Burke TR Jr, Yao Z, Gao Y, Wu JX, Zhu X, Luo JH, Guo R, Yang D*. N-Terminal carboxyl and tetrazole-containing amides as adjuvants to Grb2 SH2 domain ligand binding. *Bioorg Med Chem* 2001 Jun;9(6):1439-45

Gao, Y., Luo, J., Yao, Z., Voigt, J., Guo, R., Zuo, H., Yang, D*. and Burke, T.R. Inhibition of Grb2 SH2 domain binding by non-phosphate containing ligands 2,4-(2-malonyl) phenylalanine as a potent phosphotyrosyl mimetic. *Journal of Medicinal Chemistry*, 43(5):911-920, 2000. (shared senior author, and my postdoc is shared first author).

Yang Z. Huang, Sandra Won, Declan W. Ali, Qiang Wang, Michael Tanowitz, Quan S. Du, Kenneth A. Pelkey, **Dajun Yang**, Wen C. Xiong, Michael W. Salter, and Lin Mei. Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses, **Neuron**, 2000 May;26(2):443-55.

Mandler, R., Wu, C., Ho, D., Sausville, E.A., Roettinger, A.J., Newman, D.J., King, C.R., **Yang, D.**, Lippman, M.E., Vitetta, E.S., Landolfi, N., Brechbiel, M.W., Waldmann, T.A. Conjugation of geldanamycin to an anti-HER2/neu monoclonal antibody augments the tumor-targeted inhibitory effects of the antibody on human breast carcinoma cells. **Journal of Nat. Cancer Inst.**, 92:1573-81, 2000.

Hijazi MM, Thompson EW, Tang C, Coopman P, Torri JA, **Yang D**, Mueller SC, Lupu R. Heregulin regulates the actin cytoskeleton and promotes invasive properties in breast cancer cell lines. **Int J Oncol** 2000 Oct;17(4):629-41

Gao Y, Wu L, Luo JH, Guo R, **Yang D***, Zhang ZY, Burke TR Jr . Examination of novel non-phosphorus-containing phosphotyrosyl mimetics against protein-tyrosine phosphatase-1B and demonstration of differential affinities toward Grb2 SH2 domains. **Bioorg Med Chem Lett** 2000 May 1;10(9):923-7.

Ya-Qiu Long, Zhu-Jun Yao, Feng-Di T. Lung, Johannes H. Voigt, C. Richter King, Terrence R. Burke, Jr., Juliet H. Luo, **Dajun Yang***, and Peter P. Roller, Structural Requirements for Tyr in the Consensus Sequence Y-E-N of a Novel Non-phosphorylated Inhibitor to the Grb2-SH2 Domain, **Biochem. Biophys. Res. Commun.**, 264:902-908, 1999.

Burke, T.R., Gao, Y., Yao, Z., Voigt, J., Luo, J., and **Yang, D***. Potent Non Phosphate-Containing Grb2 SH2 Domain Inhibitors. **Peptide Science**, 20: 49-52, 1999.

Gao, Y., Yao, Z., Voigt, J., Luo, J., **Yang, D***. and Burke, T.R. Novel phosphotyrosyl mimetics for the preparation of potent small molecule Grb2 SH2 domain inhibitors. "Peptides for the New Millennium: proceedings of the 16th American Peptide Symposium", G. B. Fields, J.P. Tam, and G. Barany (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 10-13, 1999.

Long, Y.Q., Lung, F.D., Voigt, J., Yao, Z., Burke, T.R., **Yang, D***, Luo, J., Guo, R., King, C.R., and Roller, P.P. High Affinity nonphosphorylated cyclic peptide inhibitors of Grb2-SH2/growth factor receptor interaction. "Peptides for the New Millennium: proceedings of the 16th American Peptide Symposium", G. B. Fields, J.P. Tam, and G. Barany (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 102-105, 1999.

Terrence R. Burke, Jr., Juliet Luo, Zhu-Jun Yao, Yang Gao, He Zhao, George W.A. Milne, Johannes H. Voigt, C Richter King and **Dajun Yang***. Monocarboxylic-based phosphotyrosyl mimetics in the design of Brb2 SH2 domain inhibitors. **Bio. Med. Chem. Lett.**, 9, 347-352, 1999.

Burke, T.R., Jr., Luo, J., Yao, Z., Gao, Y., Zhao, H., Mine, G., Voigt, J.H., King, C.R., and **Yang, D***, "Monocarboxylic-based phosphotyrosyl mimetics in the design of Brb2 SH2 domain inhibitors." **Bio. Med. Chem. Lett.**, 1999, 9, 347-352.

Yang, D., and Wang, S., "Small Molecule Antagonists Targeting Growth Factors/Receptors. **Current Pharmaceutical Design**, June, 1997, 3, 335-354.

Yang, D.*, Kuan, C., Payne, J., Kihara, A., Pierce, J.H., Pastan, I., and Lippman, M.E., "Recombinant Heregulin-*Pseudomonas* Exotoxin Fusion Proteins: Interactions with the Heregulin Receptors and Antitumor Activity *in vivo*." **Clin. Cancer Res.**, 1998, 4:993-1004.

Harris, L., Tang, C., **Yang, D.**, Harris, A., and Lupu, R., "Induction of sensitivity to doxorubicin and etoposide by transfection of MCF-7 breast cancer cells by heregulin." **Clinical Cancer Res.**, 1998, 4:1005-1012.

RELATED ORAL PRESENTATIONS:

89th Annual Meeting of American Association for Cancer Research, New Orleans, USA, "Discovery of small molecule antagonists of heregulin/heregulin receptors through the computer-based pharmacophore 3-D database search", Pharmacology and Experimental Therapeutics, Novel Agents and Targets Minisymposium, March 30, 1998

RECENT RELATED ABSTRACTS

Dajun Yang, Yan Ling, Istvan Enyedy, Jingson Wang, Xiaofeng Zhu, Zhujun Yao, and Shaomeng Wang. Selective Inhibition of ErbB-2 (Her-2/neu) Kinase and Anti-tumor Activity with a Novel Class of ErbB-2 Specific Kinase Inhibitor. **Late-Breaking Abstract**. 11th NCI-EORTC-AACR Symposium on New Drugs in Cancer Therapy, Amsterdam, November, 2000.

Ribo Guo, Yan Ling, Juliet Luo, Zhu-Jun Yao, Hong Zuo, Yang Gao, James Kelley, Johannes H. Voigt, C. Richter King, Terrence R. Burke, Jr. and **Dajun Yang**. Inhibiting Grb2 SH2 Domain Interactions is Cytostatic and Enhances sensitivity to Chemotherapeutic Drugs in Human Breast Cancer Cells Overexpressing Her-2/neu. *Proc. Amer. Assoc. Cancer Res.*, Vol. 41, #3068, pp481, 2000. (Poster Discussion)

Burke, T.R., Gao, Y., Yao, Z., Voigt, J., Luo, J., and **Yang, D.** Potent Non Phosphate-Containing Grb2 SH2 Domain Inhibitors. "Peptide Chemistry 1999: proceedings of the 36th Japanese Peptide Symposium", pp 100-104, 1999.

Wang, S., Guo, R., Zuo, H., Wang, J.S., Lippman, M.E., and **Yang, D.** ErbB-3 and ErbB-4 Receptors Ligand Heregulin Selective Small Molecule Antagonists for Breast Cancer Therapy 7th SPORE Investigators' Workshop, NCI, Rockville, July 11-13, 1999. P12.

Wang, S., Guo, R., Tan, J., Payne, J., Milne, G.W.A., Lippman, M.E., and **Yang, D.** Discovery of Small Molecule Antagonists of Heregulin/Heregulin Receptors Through the Computer-based Pharmacophore 3-D Database Search. *Proc. Amer. Assoc. Cancer Res.*, Volume 39, #1209, pp77, 1998.

S.Wang, J.Tan, J.Payne, G.W.A. Milne, M.E.Lippman and **D.Yang*** Discovery of Small Molecule Antagonists of Heregulin/Heregulin Receptors Through the Computer-based Pharmacophore 3-D Database Search. 5th Annual SPORE Meeting, July 1997.

S. Wang, J. Tan, J. Payne, G.W.A. Milne, M. E. Lippman and **D. Yang*** Discovery of Small Molecule Antagonists of Heregulin/Heregulin Receptors Through the Computer-based Pharmacophore 3-D Database Search. 4th Annual International Conference on New Advances in "Peptidomimetics and Small Molecule design", March, 1997.

*Those are related publications in the same target use small molecule approaches or biological studies of heregulin.

- patents and licenses applied for and/or issued;
- 1. PCT/US97/21474, Title: Heregulin antagonists and methods for their use
Inventors: **Yang, D.**, Wang, S., Lippman, M.E., and Kozikoski, A.
- degrees obtained that are supported by this award;
Not applicable.
- development of cell lines, tissue or serum repositories;
Not applicable.
- informatics such as databases and animal models, etc;
Not applicable.
- funding applied for based on work supported by this award;
- 1. SPORE (Specialized Program of Research Excellence) Breast Cancer, NIH 1P50CA5818
Role: **Developmental Project Leader**
Title: Small molecule antagonist of heregulin and heregulin receptors for breast cancer
Funding Agent: National Cancer Institute/NIH, 1998-2000, annual direct \$50,000
This is a developmental project focus on the testing the novel small molecule antagonists of heregulin as anti-tumor and combination therapy in breast cancer.
- 2. Innovative Research and Development Award (IDEA) grant
Co-Principal Investigator (with Dr. Shaomeng Wang)
Title: Structure based design of erbB-2 selective small molecule kinase inhibitors
US Army Medical Research and Materiel Command, BC990340
2000-2003, annual direct \$75,000, annual indirect \$ 40,000
This grant is to discover and design the small molecule inhibitors that selectively inhibit the erbB-2 kinase activity.
- employment or research opportunities applied for and/or received on experiences/training supported by this award.
Not Applicable.

CONCLUSIONS: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address

the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the annual and final reports.

The EGF receptors (or *erbB*) are prototypes for a family of structurally related transmembrane proteins that play a role in pathogenesis of cancer. Members of this family including the EGFR, *erbB-2/neu*, *erbB-3* and *erbB-4* gene, are overexpressed in at least 60-70% of breast cancers. HRG has been found to stimulate proliferation of mammary epithelial cells both *in vitro* and *in vivo* and elevated expression in human breast cancers is associated with tumor progression and poor prognosis.

We have developed a novel, structure-based strategy towards the discovery of small, non-peptidal molecules as potential specific HRG antagonists. In this approach, pharmacophore (spatial arrangement of functional groups) models were constructed based upon the crucial residues in HRG that may be responsible for binding to its receptors, as well as the chemical nature and the three dimensional (3D) geometry of these residues in the 3D structure of HRG. An effective, computerized 3D-database pharmacophore search technique was then employed to identify small molecules in the NCI 3D-database that resemble these pharmacophores. In essence, this search detected compounds whose 3D structures mimic the binding domain of the HRG. Thus far, three classes of **lead compounds** have been discovered as specific HRG antagonists in receptor binding competition, HRG-induced phosphorylation assays. Addition of excessive amount of HRG can reverse the inhibitory effects mediated by the small molecules. These are the results of testing 105 candidates, selected from the 3D-database pharmacophore search results of 206,000 compounds in the NCI 3D-database. To date, there is no report that small molecules can act as specific antagonists of HRG.

Targeted disruption of a clinically relevant, oncogenic protein with small molecules is a new and attractive strategy. Our 3D-database pharmacophore search technique, and functional assay of the lead compounds is an unique and effective approach. Several small molecule compounds that inhibits the PDGF or VEGF receptor kinase activity has entered phase I or phase II clinical trials for the treatment of solid tumors. Our program has unique advantages. First, several promising lead compounds have already been discovered. Second, these lead compounds are small, non-peptidal, drug-like molecules. In fact, they are all natural products. Third, we have assembled a team with extensive experience in molecular modeling, drug design, cell and molecular biology, and pre-clinical studies in breast cancer research. This provides us unparalleled strength for discovery of lead compounds using our effective approaches and advancement of lead compounds into pre-clinical and phase I clinical studies.

REFERENCES: List all references pertinent to the report using a standard journal format such as *Science*, *Military Medicine*, etc.

- Alimandi M; Romano A; Curia MC; Muraro R; Fedi P; Aaronson SA; Di Fiore PP; Kraus MH Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene* 1995 May 4;10(9):1813-21
- Alley, M., Scudiero, D., Monks, A., Hursey, M., Czerwinski, M., Fine, D., Abbott, B., Mayo, J., Shoemaker, R., and Boys, M. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.*, 48:589-601, 1988.
- Barbacci EG; Guarino BC; Stroh JG; Singleton DH; Rosnack KJ; Moyer JD; Andrews GC. The structural basis for the specificity of epidermal growth factor and heregulin binding. *J Biol Chem* 1995 Apr 21;270(16):9585-9

- Bridges, A.J., The epidermal growth factor receptor family of tyrosine kinases and cancer: can an atypical exemplar be a sound therapeutic target? *Current Med. Chem.*, 3:167-194, 1996.
- C. Tang, Perez C, Grunt T., Waibel C., C. Cho, and R. Lupu. Involvement of *heregulin* B2 in the acquisition of the hormone-independent phenotype of breast cancer cells. *Cancer Res.*, July, 1996.
- Carraway III, K.L. and Cantley, L.C. a new acquaintance for erbB-3 and erbB-4: a role for receptor heterodimerization in growth signaling. *Cell*, 78:5-8, 1994.
- Carraway, K.L., 3rd, Sliwkowski, M.X., Akita, R., Platko, J.V., Guy, P.M., Nuijens, A., Diamonti, A.J., Vandlen, R.L., Cantley, L.C., and Cerione, R.A. The *erbB3* gene product is a receptor for heregulin. *J. Biol. Chem.* 269: 14303-14306, 1994.
- Carraway, K.L., Weber, J.L., Unger, M.J., Ledesma, J. Yu, N., Gassman, M. and Lai, C. Neuregulin-2, a new ligand of erbB-3/erbB-4 receptor tyrosine kinases. *Nature*, 387:512-516, 1997
- Chang, H., Riese, D.J., Gilbert, W., Stern, D.F. and McMahon, U.J. Ligands for erbB-family receptors encoded by the neuregulin-like gene. *Nature*, 387:509-511, 1997.
- Chen X., Levkowitz G., Tzahar E., Karunagaran D., Lavi S., Ben-Baruch N., Leitner O., Ratzkin B. J., Bacus, S. S., and Yarden, Y. An immunological approach reveals biological difference between the two NDF/hergulin receptors, erbB-3 and erbB-4. *J. Biol. Chem.* 271:7620, 1996.
- D. Yang, C. Kuan, J. Payne, A. Kihara, J.H. Pierce, I. Pastan and M.E. Lippman. Recombinant Heregulin-*Pseudomonas* Exotoxin Fusion Proteins: Interactions with the Heregulin Receptors and Antitumor Activity *in vivo*. *Clin. Cancer Res.*, 4:993-1004, 1998
- D. Yang, C. Loudon, D.S. Reinhold, S.K. Kohler, V.M. Maher, and J.J. McCormick. Malignant transformation of human fibroblast cell strain MSU-1.1 by (+)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. *Proc. Natl. Acad. Sci. USA*, 89, 2237-2241, 1992.
- De Potter IY, Poumay Y, Squillace KA, Pittelkow MR. Human EGF receptor (HER) family and heregulin members are differentially expressed in epidermal keratinocytes and modulate differentiation. *Exp Cell Res* 2001 Dec 10;271(2):315-28
- Lupu, R., Colomer, R., Zugmaier, G., Shepard, M., Slamon, D., and Lippman, M.E. Direct interaction of a ligand for the erbB2 oncogene product with the EGF receptor and p185erbB2. *Science*, 249: 1552-1555, 1990.
- Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S., and Fischbach, G.D. RIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell*, 72:801-815, 1993.
- Gasparini, G., Gullick, W.J., Maluta, S., Palma, P.D., Caffo, O., Leonardi, E., Boracchi, P., Poza, F., Lemoine, N.R. and Bevilacqua, P. C-erbB-3 and c-erbB-2 protein expression in node-negative breast carcinoma--an immunohistochemical study. *Eur. J. Cancer*, 30A:16-22, 1994.
- Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., et al. Identification of heregulin, a specific activator of p185erbB2. *Science*. 256: 1205-1210, 1992.
- Hong, H.; Neamati, N.; Wang, S.; Pommier, Y.; Milne, G.W.A. Discovery of Human Immunodeficiency Virus Type 1 Integrase Inhibitors by Pharmacophore Searching, *J. Med. Chem.* 1996 (submitted).
- Jacobsen, N.E., Abadi, N., Sliwkowski, M.X., Reilly, D., Skelton, N.J., and Fairbrother, W.J. High-resolution solution structure of the EGF-like domain of heregulin- α . *Biochemistry*, 35:3402-3417, 1996.
- Jones, F.E., Joseph Jerry, D., Guarino, B.C., Andrews, G.C., and Stern, D.F. Heregulin induces *in vivo* proliferation and differentiation of mammary epithelial into secretory lobuloalveoli. *Cell Growth and Diff.*, 7:1031-1038, 1996.
- Jones, J.T., Ballinger, M.D., Pisacane, P.I., Lofgren, J.A., Fitzpatrick, V.D., Fairbrother, W.J., Wells, J.A., and Sliwkowski, M.X., Binding interaction of the heregulin EGF domain with erbB-3 and erbB-4 receptors assessed by alanine scanning mutagenesis. *J. Biol. Chem.* 273:11667-11674, 1998.
- Kita, Y.A., Barff, J., Luo, Y., Wen, D., Brankow, D., Hu, S., Liu, N., Prigent, S.A., Gullick, W.J., and Nicolson, M. Ndf/hergulin stimulates the phosphorylation of her3/erbB3. *FEBS Lett.* 349: 139-143, 1994.

- Krane, I., and Leder, P. NDF/hereregulin induces persistence of terminal end buds and adenocarcinomas in the mammary glands of transgenic mice. *Oncogene*. 12:1781-1788, 1996
- Le XF, Marcelli M, McWatters A, Nan B, Mills GB, O'Brian CA, Bast RC Jr. Heregulin-induced apoptosis is mediated by down-regulation of Bcl-2 and activation of caspase-7 and is potentiated by impairment of protein kinase C alpha activity. *Oncogene* 2001 Dec 13;20(57):8258-69
- Lemoine, N.R., Barnes, D.M., Hollywood, D.P., Hughes, C.M., Smith, P. Dublin, E., Prigent, S.A., Gullick, W.J., and Hurst, H.C. Expression of C-erbB-3 gene product in breast cancers. *Br. J. Cancer*, 66: 1116-1121, 1992.
- Leung, H.Y., Weston, J., Gullick, W.J. and Williams, G. A potential autocrine loop between heregulin-alpha and erbB-3 receptor in human prostatic adenocarcinoma. *British J. of Urology*, 79:212-216, 1997.
- Lewis GD, Lofgren JA, McMurtrey AE, Nuijens A, Fendly BM, Bauer KD, and Sliwkowski MX. Growth regulation of human breast and ovarian tumor cells by heregulin: evidence for the requirement of erbB-2 as a critical component in mediating heregulin responsiveness. *Cancer Res.*, 56:1457, 1996.
- Li, S.L., Gao, J., Satoh, T., Friedman, T.M., Edling, A.E., Koch, U., Choksi, S., Han, X., Korngold, R., and Huang, Z. A computer screening approach to immunoglobulin superfamily structures and interaction: discovery of small non-peptidic CD4 inhibitors as novel immunotherapeutics. *Proc. Natl. Acad. Sci. USA*, 94:73-78, 1996
- Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J.B., Hsuan, J.J., Totty, N.F., Otsu, M., McBurney, R.N., Waterfield, M.D., Stroobant, P., and Gwynne, D. Glial growth factors are alternatively spliced erbB-2 ligands expressed in the nervous system. *Nature*, 362:312-318, 1993.
- Mason, W., Malkin, M., Leiberman, F., Cropp, G., and Hannah, A. Pharmacokinetics of SU101, a novel signal transduction inhibitor, in patients with recurrent malignant glioma. *Proc. Amer. Assoc. for Cancer Res.*, 37:166, #1145, 1996.
- Milne, G.W.A., Nicklaus, M.C., Wang, S., Driscoll, J. and Zaharevitz, D. The NCI drug information system 3D database. *J. Chem Inf. Comput. Sci.*, 34:1219-1224, 1994.
- Minncione, G.; Kannan, B.S., Colletta, G., Giardiello, F., Sliwoski, M., Yarden, Y., Normanno, N., Kim, and Salomon, D.S. Enhanced expression of heregulin in c-erbB-2 and c-H-ras transformed mouse and human mammary epithelial cells. *J. Cell. Biochem.*, 60:437-446, 1996.
- Myers, R.B., Srivastava, S., Oelschlager, D.K. and Grizzle, W.E. Expression of p160erbB-3 and p185erbB-2 in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. *J. Natl. Cancer Inst.* 86:1140-1145, 1994.
- Nagata K; Kohda D; Hatanaka H; Ichikawa S; Matsuda S; Yamamoto T; Suzuki A; Inagaki F Solution structure of the epidermal growth factor-like domain of heregulin-alpha, a ligand for p180erbB-4. *EMBO J* 1994 Aug 1;13(15):3517-23
- Normanno N; Kim N; Wen D; Smith K; Harris AL; Plowman G; Colletta G; Ciardiello F; Salomon DS Expression of messenger RNA for amphiregulin, heregulin, and cripto-1, three new members of the epidermal growth factor family, in human breast carcinomas. *Breast Cancer Res Treat* 1995 Sep;35(3):293-7
- Normanno N; Qi C.F., Gullick W.J., Persico G., Yarden Y., Wen D; Plowman G; Kenney N., Johnson G., Kim N., Brandt R., Martinez-Lacaci I., Dickson R.B., and Salomon DS Expression of amphiregulin, cripto-1, and heregulin in human breast cancer cells. *Int. J. Onco.* 2:903, 1993.
- Peles, E., and Yarden, Y. Neu and its ligand: from an oncogene to neural factors. *Bioessays*, 15:815-824, 1993.
- Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Levy, R. B., and Yarden, Y. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell*. 69: 205-216, 1992.

- Plowman, G., Culouscou, J., Whitney, G., Green, J., Calton, G., Foy, L., Neubauer, M., and Shoyab, M. Ligand-specific activation of HER4/p180^{erbB-4}, a fourth member of the epidermal growth factor receptor family. *Proc. Natl. Acad. Sci. USA.*, 90:1746-1750, 1993.
- Plowman, G.D., Green, J.M., Culouscou, J.M., Carlton, G.W., Rothwell, V.M., and Buckley, S. Heregulin induces tyrosine phosphorylation of her4/p180^{erbB-4}. *Nature*, 366: 473-475, 1993.
- Quinn, C.M., Ostrowski, J.L., Lane, S.A., Loney, D.P., Teasdale, J. And Benson, F.A. c-erbB-3 protein expression in human breast: comparison with other tumor variables and survival. *Histopatho.*, 25:247-252, 1994.
- R. Lupu, M. Cadillo, C. Cho, L. Harris, M.Hijaz, C. Pereze, K. Rosenberg, D. Yang, and C. Tang. The significance of *heregulin* in breast cancer tumor progression and drug resistance. *Breast Cancer Res. & Treat.* 1996, 38:57-66.
- Ruggiero, M., Wang, L.M., and Pierce, J. H. Mitogenic signal transduction in normal and transformed 32D hematopoietic cells. *FEBS*, 291, 203, 1991.
- Satph, T., Uehara, Y., Kaziro, Y. Inhibition of interleukin 3 and granulocyte-macrophage colony-stimulating factor stimulated increase of active ras.GTP by herbimycin A, a specific inhibitor of tyrosine kinases. *J. Biol. Chem.*, 267:2537-2541, 1992.
- Schaefer, G., Fitapatrik, V.D., and Sliwkowski, M.X. (-heregulin: a novel heregulin isoform that is an autocrine growth factor for the human breast cancer cell line, MDA-MB-175. *Oncogene.*, 1385-1394, 1997.
- Shawver LK, Schwartz DP, Taylorson LT, Lonhi MP, Jacobs, J.S., Powell, T.J., and Hirth, K.P. SU101, a potent inhibitor of PDGF-mediated signaling, inhibits growth of a wide variety of tumor types in vivo. *Proc. Amer. Assoc. for Cancer Res.*, 37:399, #2721, 1996.
- Shuker, S.B., Haiduk, P.J., Meadows, R.P. and Fesik, S.W. Discovering high-affinity ligands for proteins: SAR by NMR. *Science*. 274:1531-1534, 1996
- Simpson, B.J., Weatherill, J., Miller, E.P., Lessells, A.M., Langdon, S.P., and Miller, W.R. C-erbB-3 protein expression in ovarian tumours. *Br. J. Cancer*, 71: 758-762, 1995.
- Sliwkowski, M.X., Schaefer, G., Akita, R.W., Lofgren, J.A., Fitzpatrick, V.D., Nuijens, A., Fendly, B.M., Cerione, R.A., Vandlen, R.L., and Carraway, K.L., 3rd. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.* 269: 146
- Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and et al ErbB-3 and erbB-4 function as the respective low and high affinity receptors of all neu differentiation factor/hergulin isoforms. *J. Biol. Chem.* 269: 25226-25233, 1994.
- Venkateswarlu S, Dawson DM, St Clair P, Gupta A, Willson JK, Brattain MG. Autocrine heregulin generates growth factor independence and blocks apoptosis in colon cancer cells. *Oncogene* 2002 Jan 3;21(1):78-86
- Visscher, D.W., Sarkar, F.H., Kasunic, T.C., and Reddy, K.B. Clinicopathologic analysis of amphregulin and heregulin immunostaining in breast neoplasia. *Breast. Cancer res. And Treatment.* 45:75-80, 1997
- Wang, S., Milne, G.W.A., Yan, X., Posey, I., Nicklaus, M.C., Graham, L., Rice, W.G. Discovery of potent, non-peptide HIV protease inhibitors through 3D-database pharmacophore searching, 39:*J. Med. Chem.* 2047-2054. 1996.
- Wang, S., Milne, G.W.A., Zaharevitz, D., Sharma, R., Marquez, V.E., Lewin, N.E. and Blumberg, P.M. The discovery of novel, structurally diverse PK-C agonists through computer 3D-database pharmacophore search. *Molecular modeling studies, J. Med. Chem.* 37:4479-4489, 1994.
- Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K.M., Birchmeier, C., and Birchmeier, W. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. of Cell Biol.*, 131:215-216, 1995
- Zhang K., Sun J., Liu N., Wen D., Chang D., Thomason A., and Yoshinaga S.K. Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. *J. Biol. Chem.* 271:3884, 1996

Zhang, D., Sliwkowski, M., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Growley, C., Brush, J., and Godowski, P. Neuregulin-3 (NRG3): a novel neutral tissue-enriched protein that binds and activates erbB-4. Proc. Natl. Acad. Sci., USA, 94:9562-9567, 1997.

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples of appendices include journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

We will mail these appendices separately if needed.

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